Assessment of the genotoxicity of Cu and Zn in raw and anaerobically digested slurry with the *Vicia faba* micronucleus test

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A B S T R A C T

Genotoxicity of Cu and Zn was assessed by use of the micronucleus (MN) test on *Vicia faba* roots. Plants were exposed to various leachates of raw and anaerobically digested pig slurry, with maximum total concentrations of 200 μM Cu and 600 μM Zn. The results indicated stabilisation of the organic matter during anaerobic digestion of the slurry and bioconversion of some phytotoxic organic compounds (e.g. phenols or *p*-cresol), but did not show a relationship between Cu and Zn concentrations and MN frequency. Exposure of *Vicia* plants to binary inorganic solutions of Cu and Zn ($CuSO_4/ZnSO_4$, 1:3) showed a significant micronucleus induction at concentrations of 40 μM Cu and 120 μM Zn and higher. When MN frequency was plotted against dissolved Cu (<0.45 μm), applied as slurry or as $CuSO_4$, a single curve was obtained. At concentrations lower than 10 μM, modulation of the genotoxic effect of Cu was found. At concentrations up to 150 μM, MN induction increased significantly, while phytotoxic symptoms appeared at higher concentrations.

Keywords:
Phytotoxicity
Leachates
Metals
Oxidative stress
Organic matter
Bioconversion

1. Introduction

In France, the volume of pig slurry produced annually is estimated at 20 000 000 m^3. Pig slurries are a good source of plant nutrients such as N, P and K, which is the reason why they are spread on land as organic fertilizer. As more than 50% of the French pig production is concentrated in Brittany (which accounts for only 5% of the country's surface area), different treatment processes have been developed to deal with the excessive application of nitrogen to the soil. Recently, in a context of sustainable development, anaerobic digestion has been receiving attention since it produces a renewable energy in the form of biogas, mainly composed of methane. Because the process of anaerobic digestion does not lead to a change in the nitrogen content of the slurry, the quantities of raw and digested slurry that are spread on land are similar [1], but digested slurry is less odorous and less concentrated in dry matter than raw slurry. Indeed, different odorous and toxic compounds such as phenols and volatile fatty acids are degraded by the anaerobic fermentation [2].

The copper and zinc content of pig slurries is elevated due to the presence of animal feed supplements [3]. In recent years it has been shown that increasing levels of copper and zinc may generate a long-term environmental risk when slurries are spread on arable land for decades, due to the accumulation of these metals in the top soil [4,5]. Cu and Zn are essential nutrients and are required in very small amounts by both plants and animals, but their accumulation in soils is toxic to plants and micro-organisms [6,7]. However, several authors have reported that anaerobic digestion implies a shift of heavy metals from mobile forms towards more stable and thus less bioavailable forms [8,9]. Such chemical properties play an important role in the toxicity of the heavy metals.

Despite the acknowledged environmental risks associated with spreading pig slurry, there is little information about the actual toxicity and the genotoxicity of effluents from these slurries. De la Torre et al. [10] studied the ecotoxicological potential of pig slurry in *Daphnia magna* and found that ammonium (NH_4^+) and copper were mainly responsible for the toxicity, as well as Zn and 4-methylphenol to a lesser extent. Diez et al. [11] gave a detailed explanation of these results in a study of the chronic ecotoxicological effects of the spreading of pig slurry on soil, by means of the *Folsomia candida* reproduction test. The effects observed did not appear to be related to the organic compounds provided by the slurry (phenols, indoles or PCB), suggesting that Cu and Zn may play an important role. Over the last decades, more than 200 short-term bioassays utilizing micro-organisms, insects or plants have been developed and used to help identify agents that pose genotoxic hazards. Among these tests, genetic toxicity bioassays have proved to be very useful in environmental monitoring and assessment of pollution. Plant assays are highly sensitive, quite easy to conduct, inexpensive, and good predictors of carcinogenicity [12]. Particularly, the *Vicia faba* micronucleus test has been shown to

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Table 1
Composition of slurry leachates and $CuSO_4$ and $ZnSO_4$ binary solutions.

		Organic Matter ($g\,L^{-1}$)	Total Cu (μM)	Total Zn (μM)	pH
Raw slurry	1%	0.2	2.0	5.5	6.48
	10%	1.8	18.3	52.7	7.6
	50%	11.0	110.0	318.4	n.d.
	100%	18.0	180.9	523.7	n.d.
Digested slurry	1%	0.1	2.1	6.5	6.31
	10%	1.2	18.8	60.6	7.25
	50%	6.1	92.9	302.2	7.86
	100%	12.2	185.5	603.7	7.7
$CuSO_4$ & $ZnSO_4$ solutions	20%	0.0	38.1	112.1	n.d.
	50%	0.0	95.3	280.3	n.d.
	100%	0.0	190.6	560.6	n.d.

be sensitive in evaluating chromosomal aberrations and assessing genotoxicity from both organic and inorganic contaminants of soil [13,14], sediment [15], organic material such as sewage sludge or composts [16], and water [17–19].

Consequently, the *Vicia faba* root-micronucleus test was selected to assess the potential genotoxicity of copper and zinc when applied as raw or anaerobically digested slurry or as $CuSO_4$ and $ZnSO_4$, taking into consideration the changes and role of the organic matter during anaerobic digestion. Due to a high and phytotoxic NH_4^+ content in pig slurries, the bean roots could not be exposed directly to the effluents. A separation was carried out on the slurries to concentrate dry matter, copper and zinc into a solid fraction which was then used to prepare different concentrations of raw or digested slurry leachates.

2. Method

2.1. Origin of slurry samples and preparation of solid fractions

Raw and digested slurries were collected at the inlet and the outlet of the anaerobic digestion plant described by Marcato et al. [20]. This 150-m^3 stirred-tank reactor treated about 11 m^3 per day of pig slurry from a farrow-to-finish herd, with a retention time of 15 days. Slurry samples were collected using a sampler [20]. In order to evaluate the genotoxic potential of Cu and Zn, a method was devised to eliminate water-soluble toxic compounds and particularly ammonia. Marcato et al. [20] showed that Cu and Zn were mainly bound to particles with a size of 3–25 μm in digested slurry. Soluble NH_4^+ represented 70% of total N in raw slurry and 82% in digested slurry. Laboratory filtration techniques failed to produce sufficient quantities of solid fractions due to clogging. Slurry samples were then ultracentrifuged using a Beckam J2-21M/E centrifuge (14 000 × *g*, 30 min) to eliminate volatile fatty acids (VFA) and NH_4^+, and to concentrate the organic matter, Cu and Zn. The resulting pellets were air-dried at 40 °C to obtain a homogeneous powder. These pellets are referred to hereinafter as raw slurry (RS) and digested slurry (DS).

2.2. Chemical analysis

The main physico-chemical characteristics of RS and DS were determined. The dry matter (DM) was measured after 24 h at 105 °C. The organic matter (OM) was considered as equivalent to the volatile solids content determined by loss-ignition at 550 °C. Extraction of heavy metals (HM) such as Cu and Zn in particular was undertaken by the use of a strong-acid digestion process. An accurately weighed sample of 1 g was digested in 40 ml of a concentrated acid mixture of HNO_3 with HCl (3:1 v/v). The samples were kept overnight at 20 °C and then heated to 160 °C for 2 h. After cooling to room temperature they were filtered at 3 μm and then adjusted to 100 ml with deionised (UHQ) water. Three extraction replicates were processed for each sample. Heavy metals (Cd, Cr, Cu, Ni, Pb and Zn) were determined by plasma optical emission spectrophotometry (ICP OES Thermo IRIS Intrepid II XDL Duo). Reagent blanks and Certified Reference Material (CRM 145 R sewage sludge) were also processed in triplicate and were analysed simultaneously for quality assurance of the analytical data.

A removal percentage was calculated as described by Møller et al. [21] to characterize the ultracentrifugation efficiency and validate the method to model original slurry compositions.

2.3. Vicia faba micronucleus test

Seeds of *Vicia faba* that had been stored at 4 °C were used for this study. The *Vicia* test was carried out according to Ma et al. [22] and El Hajjouji et al. [23]. Dry seeds of *Vicia faba* were soaked for 24 h in deionised water, the seed coats were removed and the seedlings were allowed to germinate between two layers of moist cotton. After 3 days, the tips of primary roots about 2–3 cm in length were cut off and secondary roots left to grow, suspended in Hoagland's solution. Five days were necessary to obtain secondary roots of suitable length (1–2 cm) for the test. Secondary roots were then treated with different test solutions for 30 h.

Four different concentrations of RS and DS were tested and five replicates were processed for each concentration. Maximal dose (100%) corresponded to the Cu and Zn concentrations in the original slurries. The other treatments consisted of solutions that were 2-, 10- and 100-fold less concentrated (Table 1). These dilutions were prepared by weighing appropriate quantities of slurry solids and adding 750 mL of Hoagland's solution. Mixtures were stirred for 24 h at room temperature and then decanted for 2 h before the supernatant was tested. The conversion of organic matter into biogas during the anaerobic digestion implied that to obtain similar total concentrations of Cu and Zn in the raw and digested slurry leachates (Table 1) different quantities of RS and DS had to be added.

Copper ($CuSO_4$) and zinc ($ZnSO_4$) were also tested to evaluate their role in micronucleus induction. Steinkellner et al. [24] found that neither $CuSO_4$ nor $ZnCl_2$ induced any statistically significant mutagenic activity in *Vicia faba* root tips. Moreover, Cu and Zn are always present simultaneously in pig slurry. Therefore, Cu and Zn were tested in binary solutions in a 1:3 mass ratio only, at concentrations corresponding to those of the slurry leachates. The $CuSO_4$ and $ZnSO_4$ mixed stock solutions were prepared with deionised water and diluted to the right concentrations in Hoagland's solution (Table 1).

For each treatment, five replicates were used: five seeds were individually exposed. Maleic hydrazide (MH, 10^{-5} M) was used as a positive control. Aerated Hoagland's solution was used as a negative control. After treatment, root tips were fixed in Carnoy's solution (glacial acetic acid/ethanol 1:3 v/v) at 4 °C for one night, transferred to 70% ethanol for storage. The root tips were then hydrolyzed in 1 M HCl at 60 °C for 6 min. Five slides were prepared for each of the five seeds. After staining the root tips with 1% aceto-orcein, the interphase cells as defined by Ma et al. [22] were scored for micronuclei at 1000 × magnification. At least 1000 cells were counted per slide, i.e. the micronucleus frequency was obtained from at least five thousand cells per seed. In order to avoid underestimation of the micronucleus frequency due to impaired cell proliferation rate, the micronucleus test was performed only on root tips with a mitotic index (MI) greater than 2% [25].

At the end of the experiment, test solutions were filtered through a 0.45-μm cellulose filter. Filtrates were analysed by ICP-OES without previous acid digestion to determine dissolved (<0.45 μm) Cu and Zn concentrations. These concentrations are referred to below as dissolved metals, while total Cu or Zn corresponds to the sum of dissolved and particle-associated metal.

2.4. Statistical analysis

Statistical analysis was performed on the collected data. The mean value of the negative and positive controls and the different groups was obtained from descriptive analysis, and one-way ANOVA was conducted to obtain *F*-values and MS errors. Dunn's test [25] was then used to determine the level of significance against the negative control values in each experimental series.

3. Results

3.1. Chemical analysis

The main physico-chemical characteristics of RS and DS samples are reported in Table 2. Marcato et al. [20] showed that the proportion of organic matter (OM) decreased by 12% in the dry matter during anaerobic digestion. Moreover, these authors demonstrated that anaerobic digestion was conservative for Cu and Zn: their concentration increased 1.8-fold in dry matter, proportionally to the

Table 2
Analysis of the pellets obtained by ultracentrifugation.

		Raw slurry	Digested slurry
Organic matter (% DM)		72.4	63.9
Heavy metals (mg kg^{-1} DM)			
	Cd	<l.d.	<l.d.
	Cr	4	4
	Cu	353	558
	Ni	6	6
	Pb	3	4
	Zn	1053	1872

loss due to bio-conversion of the organic matter into biogas. Table 2 indicates that solid fractions from ultracentrifugation are quite representative of the original slurries since the OM content in the DM decreased by 12% between RS and DS, while Cu and Zn are, respectively, 1.6- and 1.8-fold more concentrated in DS.

The recovery efficiencies (over 80% for Cu and 90% for Zn) confirmed the relevance of using the ultracentrifugation pellets to represent the original slurries (Fig. 1). Recovery efficiency was lower for DM, probably due to the loss of dissolved inorganic and organic compounds that cannot be separated even with 0.45-μm filtration [26].

3.2. Micronucleus test of slurry leachates

The genotoxic activities of the slurry leachates are shown in Table 3. For raw slurry concentrations higher than 10% (i.e. 50 and 100%) and for the 100% digested slurry, blackening of the root tips occurred and the loss of mitosis was observed. Under these conditions micronuclei were not quantified. The difference in toxicity observed between RS and DS at the 50% dose could only be due to the OM content, since the concentrations of Cu and Zn were similar. Phenols in the raw slurry that had been shown to be toxic or genotoxic [10–23] were degraded during anaerobic digestion [27,28], which may explain the decrease of the toxic effect of the OM after treatment.

For the lowest concentrations of raw slurry (1 and 10%), a significant increase in micronucleus frequency was recorded, compared with the negative control (Table 3). For the digested slurry, a significant increase in micronucleus induction was also observed whatever the concentration. The micronucleus frequencies were very close between the different treatments, ranging between 8.8 and 10.2 micronuclei per 1000 cells. The maximum micronucleus frequency was observed for the 10%-concentration of digested slurry (14.3 ± 4).

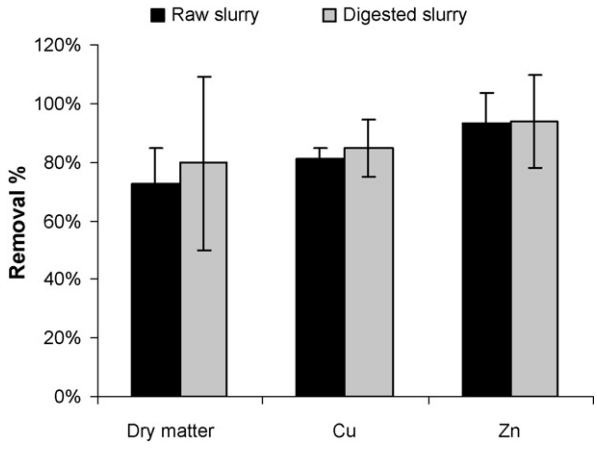

Fig. 1. Ultracentrifugation recovery efficiencies.

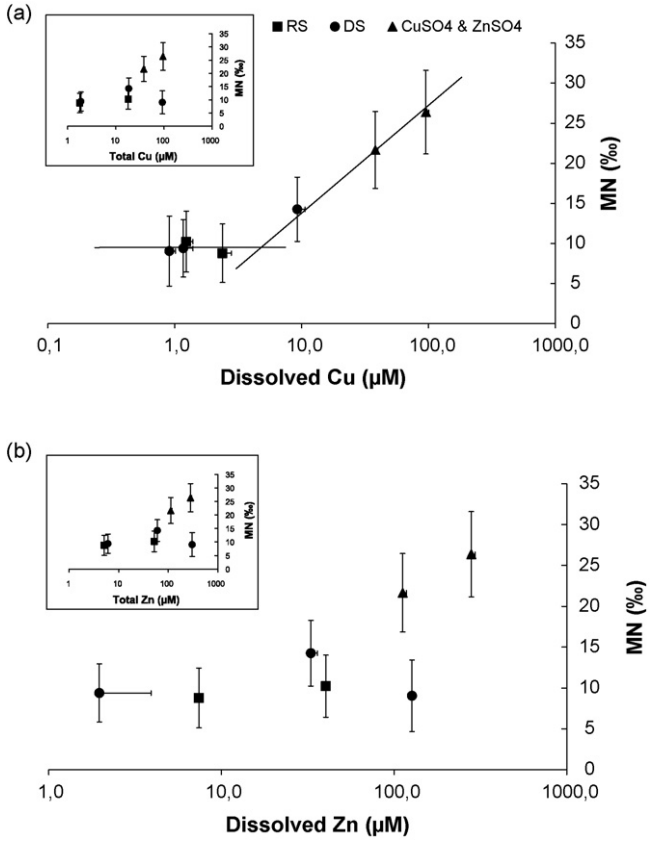

Fig. 2. Relationships between MN frequency in *Vicia faba*-root and copper (a) or zinc (b) concentration.

3.3. Micronucleus test of Cu and Zn inorganic solutions

The results of the *Vicia faba* micronucleus assay with inorganic solutions of Cu and Zn are shown in Table 3. All concentrations caused the formation of MN, peaking at 26.4‰ for the 50% treatment (95 μM Cu and 280 μM Zn). This value is 3-fold higher than the MN frequency obtained with the 50% DS leachate, suggesting that the OM present in DS protects the roots. Indeed, Zn and particularly Cu remain closely bound to the organic matter in pig slurry [20–26].

At concentrations higher than 150 μM Cu and 500 μM Zn (i.e. for the most concentrated solution, 100%), the mitotic index was significantly reduced (below 2%) and the micronucleus test was not used to monitor toxicity.

3.4. Relationship between Cu and Zn concentrations and MN induction

No correlation was found between the level of total Cu applied (as raw or digested slurry or as $CuSO_4$) and the MN frequency (Fig. 2). MN induction was fairly constant for all slurry treatments and, for a given total Cu concentration (100 μM) the MN frequency was higher when Cu was applied as $CuSO_4$, indicating that slurry-derived Cu is less detrimental to *Vicia* roots than $CuSO_4$. In contrast, when MN frequency was plotted against the concentration of dissolved Cu, a single curve was obtained for all treatments (Fig. 2). For concentrations below 10 μM, the MN frequency increased slightly in comparison with the negative control. Then, an increase of the MN frequency was observed, the response becoming statistically significant and increasing with increasing levels of dissolved Cu. Toxicity symptoms appeared at concentrations of 150 μM and higher, and MN frequency was not assessed.

Table 3
Mitotic index and micronucleus frequencies in *Vicia faba* root exposed to different RS and DS leachates and inorganic solutions.

		Mitotic index/100 cells ± SD ($n = 5$)	MN/1000 cells ± SD ($n = 5$)
Slurry leachates			
Negative control (Hoagland's solution)		9.8 ± 1.5	3.9 ± 1.7
Positive control (MH 10^{-5} M)		6.3 ± 3.0	19.0 ± 5.7*
Raw slurry	1%	11.14 ± 3.6	8.8 ± 3.6*
	10%	9.74 ± 0.8	10.2 ± 3.8*
	50%	n.d.	n.d.
	100%	n.d.	n.d.
Digested slurry	1%	10.4 ± 2.2	9.4 ± 3.6*
	10%	11.1 ± 0.9	14.3 ± 4.0*
	50%	14.0 ± 3.8*	9.0 ± 4.3*
	100%	n.d.	n.d.
$CuSO_4$ & $ZnSO_4$ solutions			
Negative control (Hoagland's solution)		5.1 ± 0.5	2.8 ± 1.7
Positive control (MH 10^{-5} M)		6.7 ± 3.1	30.6 ± 8.7*
	20%	8.4 ± 1.2*	21.7 ± 4.8*
	50%	6.3 ± 1.3	26.4 ± 5.2*
	100%	1.1 ± 0.5	n.d.

* $p < 0.05$ against the NC, not detected (n.d.).

Such a relation could not be found between MN frequency and dissolved Zn concentration (Fig. 2). Actually, dissolved Zn concentration was dependent on total Zn concentration, which was not observed for Cu due to the high affinity of this metal to organic matter. These results indicate that the difference in MN induction between the organic and inorganic sources of Cu can be attributed to the difference in dissolved Cu content in the exposure solutions, which can be considered as the bioavailable Cu fraction.

4. Discussion

In this study, the genotoxicity of raw slurry (RS) and digested slurry (DS) was assessed by use of the *Vicia faba* MN assay. Since Tiquia et al. [29] demonstrated that ammonia and copper were the main toxic compounds of pig slurry, the level of ammonia was drastically lowered in a centrifugation step that concentrated the copper and zinc in a particulate organic fraction (Table 2). Due to the high concentrations of organic matter, copper and zinc, different dilutions of RS and DS were tested. In each dilution, total Cu and Zn concentrations were similar for DS and RS.

For the RS-50%, RS-100% and DS-100% treatments, a loss of mitosis and a blackening of the root tips were observed after exposure (data not shown), illustrating the acute toxicity of these slurry concentrations. Further physico-chemical characteristics of slurry such as pH (7.8) and total organic matter are in line with these toxic effects. Pig slurries carry compounds other than copper and zinc, like phenols [10] and volatile fatty acids (VFA). Consequently, the toxicity observed at these concentrations was not only due to the concentrations of total Cu and Zn but also to the organic components in the slurry. Moreover, these results indicate a beneficial effect of anaerobic digestion on toxic organic elements like phenol or *p*-cresol [27,28], but also show a genotoxic potential of these slurries.

To investigate the genotoxic effects of Cu and Zn on *Vicia faba* roots, concentrations of total Cu and Zn similar to those present in slurry leachates were tested with water-soluble salts ($CuSO_4$ and $ZnSO_4$) without addition of organic matter. At concentrations higher than 100 μM Cu and 300 μM Zn (i.e. for the 100% treatment), the mitotic index was lower than 2%, indicating acute toxicity directly associated with the metals. The results for less concentrated solutions (20 and 50%) clearly indicated that Cu and Zn significantly increased the MN frequency in *Vicia faba* root tips. The comparison of these data with the RS and DS results (Table 3) suggests that: (i) anaerobic digestion decreased the acute toxicity of a slurry, confirming the toxicity of the organic matter; (ii) the genotoxicity of these slurries was strongly associated with the presence of Cu and Zn. These results are in contrast to the work by Steinkellner et al. [24]. These authors found that copper was not mutagenic in a concentration range from 0.5 to 2 mM in any of the three plant bioassays tested: *Tradescantia*, *Allium cepa* and *Vicia faba*. In the same work, the authors assessed that Zn was the weakest mutagen tested; whatever the concentration in a range from 250 to 2000 mM, the response was negative in the *Vicia faba* assay. Three major differences between our work and the study of Steinkellner et al. [24] should be noted to explain the observed differences: (i) the tested metal concentrations were 1000- to 10000-fold lower in our work; (ii) in our experimental conditions, *Vicia faba* root s were exposed for 30 h instead of 2 h; (iii) metals were tested in binary solutions in the present work and separately in the study by Steinkellner et al. [24].

Similarly contrasting results were found by different authors studying the toxicity of unary and binary solutions of Cu and Zn to *Vibrio fischeri*. Unary solutions of Cu or Zn were always found to decrease the bioluminescence of *Vibrio fischeri*. However, when testing binary solutions, Ince et al. [30] and Fulladosa et al. [31] observed a merely additive effect, while at lower concentrations Utgikar et al. [32] showed a synergistic effect of Cu and Zn. Results obtained with binary solutions of Cu and Zn clearly demonstrated the genotoxicity of this combination of metals. By exposure to slurry leachates, this genotoxic potential could not be easily observed due to the toxicity of various organic compounds. Considering all the treatments (slurry leachates and metallic solutions) that did not show toxicity, no relation could be determined between total Cu or total Zn concentrations and the MN frequency. Earlier work (data not shown) involving exposure of *Vicia faba* roots to $ZnSO_4$ solution, indicated that Zn induced a significant increase of MN frequency when the roots were exposed to $ZnSO_4$ in a concentration range from 500 to 3800 μM. At levels higher than 3800 μM, the mitotic index (MI) was reduced and became lower than 2%. The results presented in Table 3 show a reduction in MI from 500 μM Zn, indicating that total Zn was probably not directly involved in micronucleus induction, in accordance with the findings of Borboa and De la Torre [33]. The effect of total Zn, applied as raw or digested slurry or as $ZnSO_4$, on MN frequency (Fig. 2) did not indicate any relation. The trend remained the same when MN frequency was plotted against dissolved Zn. In contrast, the relationships found between dissolved Cu and MN frequency underlined the potential role of this metal in MN induction. Our data show that for concentrations up to 7.5 μM,

and in the presence of Zn, a significant dose-dependent effect was observed.

The *Vicia faba* MN test is a very sensitive and useful method that allows detection of both clastogenic and aneugenic effects [23–34]. Micronuclei are the results of chromosome breaks (or mitotic anomalies) that require a passage through mitosis to be recognisable. The molecular mechanism of DNA breakage is not yet clearly understood. Although it does not constitute the only genotoxic pathway, oxidative stress was described as being mainly implied in DNA-damage formation [35]. Copper is a common cofactor for many enzymes, including oxidases and oxygenases. Like iron, copper acts as a catalyst in the formation of reactive oxygen species and catalyzes peroxidation of membrane lipids. Relationships between dissolved copper and MN frequency are then supported by oxidative-stress mechanisms, and more particularly by the catalytic role of Cu in the Fenton reaction and the production of the hydroxyl radical $OH^{\bullet}$, which is considered to be the ultimate reactive oxygen species to interact with DNA. This radical attacks DNA on the sugar residue and induces DNA fragmentation, base loss and strand breaks with a terminal residue sugar fragment [36]. Babar Ali et al. [37] showed that *Panax ginseng* could grow under Cu stress (5–25 μM) by modulating the antioxidant defence mechanisms for combating Cu-induced oxidative stress. At higher concentrations (50 μM), antioxidant enzyme activities declined and the concentrations of toxic reactive oxygen species (H_2O_2, $O_2^{\bullet -}$) increased in roots. Such an inhibition of the antioxidant defence system can lead to clastogenicity and MN production. Although the genotoxicity of the Cu and Zn ions is not clearly established, a possible synergistic effect between Cu and Zn could be proposed [33]. It is assumed that Zn interferes with DNA-repair processes in mammals via O^6-alkylguanine-DNA-alkyltransferase and ligase I activities [38,39]. Zn could then potentiate the genotoxic effect of Cu by inhibiting the DNA-repair process.

Finally, the *Vicia faba* micronucleus test demonstrates the genotoxicity of pig slurry, which was strongly associated with the presence of Cu and Zn. In addition, it appeared that dissolved Cu was directly associated with the genotoxic effect of these slurries. Zn could also contribute to this effect via an indirect mechanism. These results also underline that anaerobic treatments have no influence on the genotoxicity of these metals. In contrast, the treatment decreased acute toxicity from organic molecules through their bioconversion. Further work is necessary to clarify the mechanism of genotoxicity of Cu and Zn in *Vicia faba* root tips.

Conflict of interest

None.

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